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BIOLOGICAL BULLETIN

A STATISTICAL STUDY OF MITOSIS AND AMITOSIS IN THE ENTODERM OF FASCIOLARIA TULIPA, VAR. DISTANS.[1]

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INTRODUCTION.

Remak's ('41) diagrammatic schema of nuclear and cell division was banished from the field of normal biology by the cytological work of the decade following its proposal. Since that time it has ever remained heresy to associate amitosis of any sort with anything else than cellular senescence, or a high grade of specialization, or intense metabolic activity. "When once a cell has undergone amitotic division it has received its death warrant," wrote vom Rath ('91), and although this assertion is now acknowledged to be extreme, its spirit is nevertheless still so firmly engrafted on biological literature and thought that the uncanonical facts claimed by Pfeffer ('99) to obtain under experimental conditions in *Spirogyra*, and by Meves ('91) under natural conditions in the testis of the salamander have been regarded more as anomalies than as contributions to our knowledge of cell division. Quite recently however Child ('04; '07 I., II., III., IV., V., VI.; '07*a*) as the result of his very careful work on the cestode *Moniezia*, and his more or less exploratory observations on representatives of almost every phylum in the animal kingdom, has forced upon cytologists so many instances in which amitosis seems to occur in normal and healthy tissues, that the significance of what he found demands serious consideration. Appeal to inadequate technical methods, to senescence, to specialization, or to pathology are insufficient. Wheeler ('89) and

[1] Contributions from the Zoölogical Laboratory, University of Michigan, No. 114.

Osborn ('04, I. and II.), have also published data that have helped to reopen the old wound. It is again debatable what part amitosis plays in normal cell differentiation, and also whether a direct nuclear division may intervene between mitotic divisions without wrecking the ability of the cell in which it occurs to have progeny capable of further differentiation. In the present paper I intend to discuss the first of these questions on the basis of determinations quantitatively as exact as the nature of the subject and material permit. The technical methods employed in fixation, staining, and sectioning, have been fully described in an earlier paper (Glaser, '05). There also will be found evidence of the adequacy of the methods used.

Developmental Stages Considered.

The developmental stages which I have considered for the purposes of this work are those of the cannibal and veliger periods. The highly interesting events of this portion of the life history of *Fasciolaria* have been described in detail (Glaser, '05) but in order to facilitate the description of both the development of the entoderm and of the nuclear phenomena exhibited by this tissue, it will be necessary to restate briefly the chief facts in the gross embryology.

The entire development of *Fasciolaria* is influenced and modified, either directly or indirectly, by the process of cannibalism. This form of embryonic nutrition seems to depend on three things: on the fact that the eggs are laid inside of capsules; that thousands of them remain unfertilized; and that the embryos within each egg-case differ markedly in age, in size, and in vigor. Given these circumstances, the most vigorous larvæ within each capsule ingest all of the infertile eggs and all of the weaklings. Stages intended to illustrate typical degrees of cannibalism are shown in the second column of Fig. 9, p. 233.

Larva I. is the earliest stage used. It shows the mouth between the two bulging external kidneys, and contains under the right one, remnants of the macromeres of the segmentation period. Farther down in the digestive tract lie two of the swallowed food-ova.

Larva II. has ingested fourteen eggs, whereas III. is a fully

gorged and distended cannibal. The lower two larvæ, IV. and V., represent stages is the development of the veliger. I have not attempted to show the ova with which they are filled, nor is it necessary at this time to discuss the external changes involved in the transformation of a cannibal into a veliger.

The Development of the Entoderm.

It will prove to be an advantage if the description of the development of the entoderm is begun at a stage earlier than I., Fig. 9. A transverse section through the earliest larva available for the present purpose is shown in Fig. 1. The section is bilaterally symmetrical and shows on the right and left, the beginnings of the external kidneys (*ex.k.*). Beneath these rudiments, is mesoderm (*mes.*) with indistinct cell boundaries, while under this layer and immediately upon the yolk, is the entoderm (*ent.*), as yet an incomplete membrane composed of a few spindle-shaped cells with extremely attenuated processes.

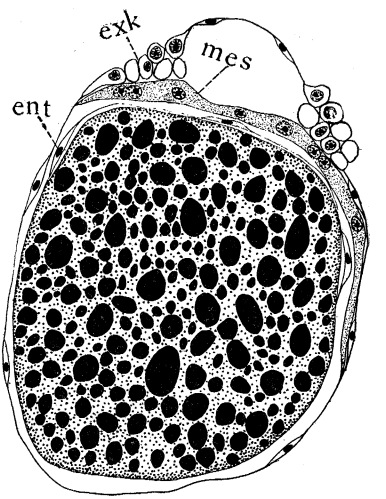


FIG. 1. A transverse section through a young pre-cannibal, showing the external kidneys (*ex.k.*); beneath these the mesoderm (*mes.*); and immediately upon the yolk, the spindle-shaped entoderm cells (*ent.*).

Fig. 2, a section cut in plane *xy* of stage I., Fig. 9, illustrates the cellular conditions met with at the beginning of cannibalism. Cell boundaries in all of the tissues except the external kidneys (*ex.k.*) are obscure. The ectoderm elsewhere is a spongy syncitium, varying considerably in consistency in different regions. The entoderm is apparently also a syncitium, but is spongy only in the anterior region *A* where it is impossible to define its limits. Ventrally *V* on the side toward the external kidney, posteriorly *P* diammetrically opposite the cap of spongy ectoderm, and dorsally *D* diammetrically opposite the external kidney, the

entoderm exhibits granulated nuclei imbedded in a granular sometimes slightly alveolar ground substance in which cell boundaries are indistinguishable. All the nuclei are surrounded by a zone in which the particles are exceedingly dense, but this

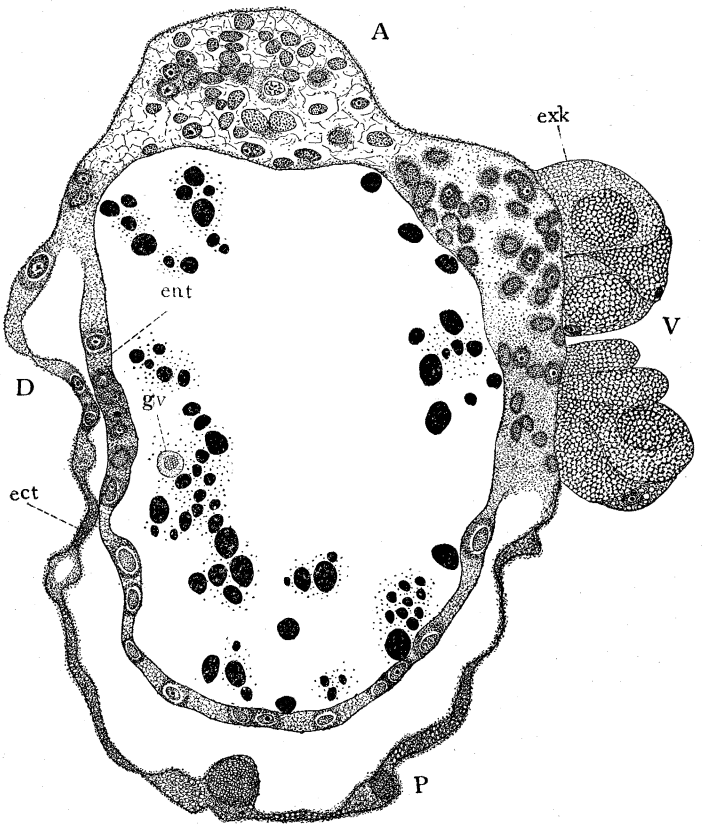


FIG. 2. A longitudinal section cut in plane *xy* of stage I., Fig. 9. On the right (ventral, *V.*) is shown the external kidney (*ex.k.*). Anteriorly *A*, where the ectoderm (*ect.*) and the entoderm (*ent.*) meet is the cap of spongy tissue described on p. 221. *G.v.* is the fragment of a germinal vesicle from one of the food ova. Note the difference between the entoderm in this stage and that characteristic of the earlier and later larvæ.

region does not always abut upon the nuclear membranes. In many cases therefore a narrow clear band devoid of granules can be seen between the nucleus and the dense zone. Often a nucleus is found to contain a nucleolus, at times surrounded by

an achromatic halo. In the lumen of the intestine are some scattered yolk spheres derived from the macromeres and the ingested food-ova. At one point, *gv*, is shown the fragment of a germinal vesicle.

When the larva has reached the distended condition of a fully gorged cannibal, the entoderm is very different from that shown in Fig. 2 (see Fig. 2). At this time the entoderm has been

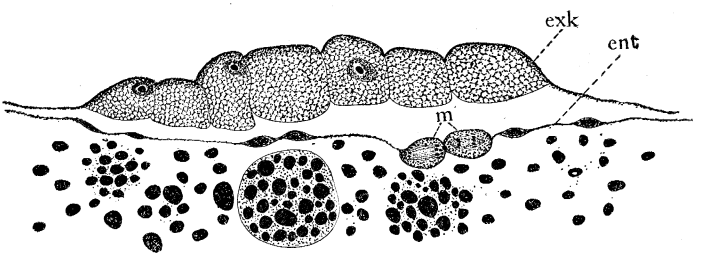


FIG. 3. Part of a section through a fully gorged cannibal, cut in a plane passing through one of the external kidneys (*ex.k.*). Notice particularly the character of the entoderm (*ent.*) the cells of which are now spindle-shaped and provided with very long and delicate processes. At *m*, two of the entoderm cells are dividing mitotically.

so highly stretched that most of its earlier characteristics have disappeared. In the first place the cells, except immediately beneath the external kidneys, are so closely crowded against the ectoderm that it is difficult to distinguish two membranes even in those regions where in earlier stages ectoderm and entoderm were separately and distinctly recognizable. The cells also are now possessed of distinct boundaries, are spindle-shaped where clearly visible and are connected by such long and finely attenuated processes that one often finds hiatuses. The presence of these breaks in the membrane lead Osborn ('04) to conclude that there is at this time not enough entoderm to enclose the food-ova. My own sections have convinced me that the hiatuses are due not to the incompleteness of the membrane in which they occur, but to its extreme delicacy. It is only preserved in exceptionally good specimens, but these together with the condition exhibited by the earlier larvæ, seem to me to warrant the conclusion that the entoderm is normally a complete membrane. The ectoderm in these fully gorged cannibals has essentially the same cellular character as the entoderm, and in perfect sections is complete.

Here too Osborn found hiatuses, but if these really occurred in the living state, it is difficult to see how a sac with holes in both its inner and outer linings could contain the eggs which these larvæ ingest.

When the fully gorged cannibals transform into veligers, the changes undergone by the entoderm are as striking as those in the external form of the larvæ. These changes lead to regional differentiation, the outcome of which is that the dorsal cells of the digestive tract come to be very unlike the ventral ones, whereas between these two zones, laterally, there are transitional cell forms. In addition to this morphological differentiation which holds true of the digestive tract from its most anterior end back to the region where it becomes identical with the digestive gland or liver, there is a well-marked physiological differentiation between the cells in the œsophageal region and those posterior to this zone. Fig. 4 shows a section, based on the study of several, through the œsophagus. The lumen of the tube is lined by comparatively small cells, provided either with several nuclei, or with lobed ones. The cytoplasmic contents of these cells are quite granular, and are often so densely crowded along the inner surfaces of the cell membranes that the nuclei in these cases seem to float in clear lakes of non-tingible cell sap.

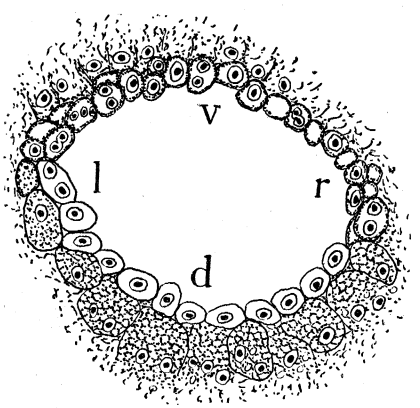


FIG. 4. A transverse section through the œsophageal entoderm of a larva in stage IV., based on the study of several sections through this region. *l*, left; *r*, right; *v*, ventral; *d*, dorsal.

The outer border of the œsophagus has a very different appearance. The cells there in many cases show unmistakable signs of disintegration, especially ventrally *v*, where often cell-fragments and quite isolated nuclei can be seen. Dorsally *d* the outermost cells are very large, polynuclear, frequently without complete cell-membranes, and their contents which are granular,

and arranged in a reticulate manner, can be seen oozing out into the "body cavity." These large dorsal cells are continuous with the liver cells.

While it may be inferred, from facts to be presented later that the cells in the posterior part of the digestive tract are engaged in the digestion and storage of food materials, those in the anterior end, on the basis of the histological evidence given above, may be assumed to be engaged in a process of internal excretion. This assumption gains in validity when we recall that an immense amount of yolk must be metabolized and also that the œsophagus is at the level of the external kidneys. Though many of the outermost cells show signs of "overwork" the disintegration which this brings about is in no sense pathological, since it

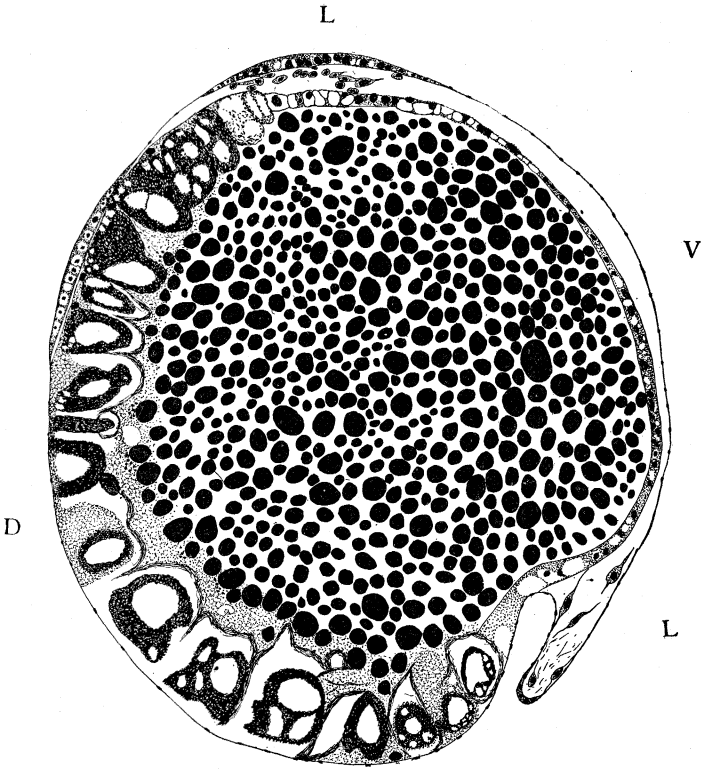


FIG. 5. A transverse section through the posterior half of a larva in stage VI. *L*, lateral; *v*, ventral; *d*, dorsal; ventrally and laterally is the comparatively undifferentiated entoderm; dorsally are the large liver cells.

occurs in all healthy larvæ, and is only a part of a normal, but highly peculiar developmental history.

Well posterior to the œsophagus, transverse sections also exhibit two very distinct kinds of entodermal elements, although one finds intermediate stages between them. Ventrally *v* and partly laterally *l* the entoderm as compared with the dorsal cells is a thin layer; the cells are granular and vacuolated, especially laterally, and except where there are transition stages into the dorsal cells, definite boundaries are not always recognizable. The striking condition of the dorsally situated liver cells is connected with digestion since they seem to serve as temporary storage places for digested or partly digested yolk. These cells are unusually large, and very remarkable in appearance. Their contents differ greatly in arrangement, and at first sight in their reactions with orange *G*, but such differences as they present in this respect are due to the density of the materials, and not to any fundamental difference in their composition. Certain irregular masses containing one or more large open spaces and many very minute ones, tinge deeply and are frequently separated by an area of considerable width from what I take to be cell boundaries. These boundaries where clearly observable are made up of exceedingly fine fibrils closely packed. Among the other cell contents seen in this region are granules of two sizes, very minute ones not always regularly distributed, and somewhat coarser ones arranged in a reticulate manner. Both of these kinds of material stain with orange *G*, though on the whole less deeply than the dense masses with the large vacant spaces. In the lumen of the digestive cavity are granules of exactly the same staining reactions as those inside of the cells and these also are arranged partly without regularity, partly in reticula. Here and there are small collections of larger granules that suggest from their grouping fragmented yolk spherules. Since all of these materials, intra-, as well as the extra-cellular, have the some staining reactions with orange *G*, I conclude that they represent stages in the digestion of yolk.

Laterally *l* and ventrally *v* the entoderm cells have a fundamentally different appearance from the liver cells; they are less definite on the whole in their outlines; are decidedly smaller in

size; contain no granules that stain with orange *G* and are occasionally almost completely filled with a vacuole, so that in certain localities I feel reasonably certain that two adjoining vacuoles often represent two cells. The nuclei of the entoderm in this region are small in comparison with those from other places.

The Nuclear Phenomena in the Entoderm.

The fact that amitosis occurs in the entoderm of *Fasciolaria* embryos, was so far as I know first definitely asserted by Osborn. "The entoderm," says Osborn ('04 I.), "is composed of cubical cells in which one finds all stages of direct division."[1] Fig. 6 represents some of these divisions. The nuclei shown in this picture were enlarged from the same sections from which Fig. 4 was compounded. *A* and *b* are removed from their cells. In one of them *a* the finely divided chromatin granules exhibit a slightly reticular arrangement and considerable condensation along the inner surface of the nuclear membrane. Here and there are larger dense collections of these granules suggesting an interrupted skein. The nucleus in question is markedly bilobed, the larger lobe having a small nucleolus, the smaller lobe a large nucleolus. Separating the two lobes incompletely is a very delicate interrupted membrane, which on close inspection was found to be composed of a dense collection of granules like those lining the inside of the nuclear membrane. I have seen these granular boundaries so frequently between the lobes of what I take to be dividing nuclei, that I conclude that cleavage is in many cases initiated by a granular plate that grows inward from the nuclear wall. Nucleus *b* is very much

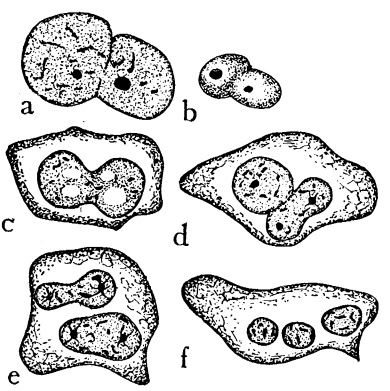


FIG. 6. Cells and nuclei from the excretory zone of the entoderm.

[1] These direct divisions were interpreted by Osborn in a later paper ('04, II.) as growth phenomena, a view supportable, as the sequel will show, by much additional evidence.

smaller than *a*, is also bilobed, and the lobes contain different sized nucleoli. *B* appears to differ from *a* in three striking details: the finely divided chromatin is not arranged in a reticulum; there are no larger chromatin bodies and the nucleoli are surrounded by large clear areas devoid of tingible material. The remaining nuclei and their cells (*c*, *d*, *e*, *f*), illustrate the conditions most commonly met with in the disintegrating cells. The cell contents, irregular masses of granules and what appear to be fibrils or strands, are crowded along the inner surfaces of the cell membranes and are separated by clear regions from the bilobed or dividing nuclei that occupy approximately the centers of the cells. These nuclei differ markedly in several respects from those already described. Their granular contents are not clearly reticulate; such large masses of chromatin as they contain are much condensed and the nucleoli often have definite chromatin radiations, a condition suggesting that all of these nucleoli are chromatin nucleoli, especially as *b* shows no other large chromatin bodies. In addition large vacuoles are often found inside of the nuclei.

The direct divisions to which I have devoted most of my attention occur in those regions of the entoderm where neither liver nor disintegrating cells are found. The nuclei there (Fig. 7) are not remarkable for size, in fact they are rather small, a condition which favors the view that they are not very active metabolically. They may or may not exhibit nucleoli, and these may or may not be surrounded by halos devoid of chromatic material. The nucleoli are usually small and their staining reaction is different from that of the other nuclear contents. The chromatin is usually scattered irregularly in the form of granules somewhat larger than those of the other amitotic entodermal nuclei. Some of the nuclei show clear spaces independent of the nucleoli, but these regions of achromatic material are not always sufficiently distinct to warrant the same interpretation for all. Some seem to be vacuolar; others are certainly not. Many of the nuclei contain two nucleoli. These may differ in size, and may lie rather close together or be separated by a considerable distance. I have never seen such nucleoli in the act of division. Among these nuclei I have found what I interpret as all possible stages

of amitosis, and the nineteen represented in Fig. 7 are cases some of which one can find in every section.

I have not been able to convince myself that there is any particular way in which these nuclei divide, on the contrary, the details of their division vary considerably and there may be others of which as yet I have no inkling. Figures such as 2, 5,

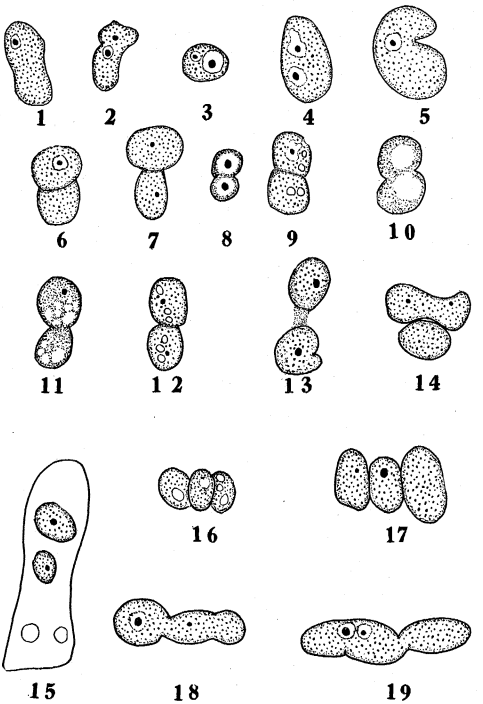


FIG. 7. Nuclei from the ventral and lateral comparatively undifferentiated entoderm in the digestive zone of stage IV. and later stages.

10, 13, suggest that the process of division may begin by the formation of a lobe, and that this lobe may then be gradually constricted off. The nuclei that one finds close together, such as 16 and 17 often differ greatly in size suggesting that the lobes from which they came may have been unequal, a condition actually observed in many instances. Number 13 is a most interesting and valuable nucleus, because it shows beyond doubt a slightly chromatic, somewhat attenuated bridge connecting

two widely separated lobes, one of which — the lower — has the nuclear membrane equally distinct throughout its circumference. This nucleus was killed in the very act of pulling apart. Other nuclei such as 6, 7, 8, 9, 11 and 12, seem to be dividing by the formation of a granular plate, such as is exhibited by some of the nuclei in Fig. 4. Others, such as 14 and 15, the latter drawn with its clearly marked cell boundaries, give no indication whatever of how the separation may have taken place. The groups 16, 17, 18 and 19, are extremely interesting as they seem to throw light on the origin of nuclear nests. Very frequently I have found three, four or five nuclei huddled together so closely that I could make out clearly no other relation between them. Often one of them is at a slightly different level from the others. In the cases under consideration the history of such nests may be read. A nucleus instead of dividing into two, in the manner of an amœba, simply elongates, and becomes lobed in two or more widely separated regions which may or may not be provided with nucleoli. These lobes later separate, and the original nucleus has divided into three or more parts, approximately equal in size or at times quite unequal. That there is nothing anomalous about this mode of division is illustrated by the nuclei in the external kidneys in which one frequently finds these conditions clearly exemplified (Fig. 8). In comparing the nuclei

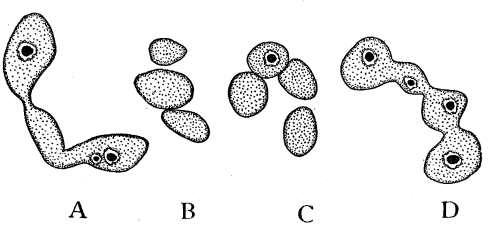


FIG. 8. Nuclei from the external kidneys where cases of multiple simultaneous division are frequent. These drawings were made from entire nuclei and show that the divisions are not dependent on the activities of the nucleoli, which may or may not be present.

just described with those in the disintegrating entoderm cells, it is clear that, excluding 17, 18 and 19, they are very much smaller in size. Furthermore, the nuclei in Fig. 7 show none of the chromatin masses exhibited by the nuclei in the disintegrating

cells, and there is no morphological indication that the nucleoli contain chromatin, as they never exhibit the radiations found so frequently in the former group. As a consequence probably of the absence of chromatin nucleoli and the other larger chromatic masses seen in the nuclei of Fig. 6, such granules as one does find, are slightly larger than the finely distributed chromatin of the nuclei in the disintegrating cells. The two kinds of nuclei therefore exhibit certain well-marked histological differences, and these differences make it comparatively easy not to mistake the one kind for the other — even in the transitional regions where both occur together — regions which I eliminated altogether from the determinations.

Difficulties.

The interpretation of the histological facts given in the preceding section offers difficulties some of which inhere in the material used, while others inhere in the subject, and would be met with no matter what animal was studied. In the first place, the technical difficulties encountered in attempting to cut serial sections were such that my series are only rarely complete and hence unsuitable for the determination of the total number of nuclei per embryo. I was able however to determine the total number of nuclei in each section, and to count the resting ones and those dividing either directly or indirectly. Each section was thus treated as an independent entity without regard to what preceded or followed it. The results therefore show that in the particular set of sections which I studied, each one treated individually, a certain number of nuclei were dividing directly and a certain number indirectly. The relative frequencies of mitosis and amitosis are in no wise altered by the imperfections alluded to.

The second difficulty that was encountered, was the physiological differentiation of the entoderm into an anterior excretory zone and a much larger posterior assimilative zone. While complicating the problem to some extent, the regulative disintegration brought on by intense excretory activity, is restricted to a very definite region, back of which nothing like it was ever observed. It is necessary of course to conclude that some of the entoderm cells are temporary larval structures, but this conclusion should not be extended so as to include the entire entoderm.

If the lining of the entire embryonic digestive tract were temporary, one should be able to find reserve elements from which at a later stage the definitive entoderm might be derived. Careful search has failed to reveal such cells. Even granting that such reserve cells do indeed exist, but that they are not sufficiently well characterized to attract attention, there are no regions in the entoderm in which amitosis is absent, and the assumption that there are reserve cells involves of necessity the belief that the definitive entoderm comes from cells like those described and figured. Since there are constant histological differences between the nuclei in the two regions under discussion, and further since the disintegrating cells are very definitely restricted, they can be eliminated from the field of inquiry by tracing them to their posterior limits and considering only cells well back of this boundary.

A third difficulty was encountered when it was found that not only is it impossible to cut mitotic figures and amitotic nuclei serially into an equal number of sections, but they cannot even be sectioned in an equal variety of planes that will reveal their true character. Actual measurements, as well as experiments with models representing direct and indirect nuclear division, show that when nuclei are equal in volume, one in anaphase can be cut in many more planes that will reveal its true mitotic character, than an amitotic nucleus of equal mass. In fact in very late stages of amitosis, stages in which the daughter nuclei are connected with one another by very small or very attenuated bridges, only planes passing through the long axis of the dumbbell shape will exhibit the true relation of the lobes. Since the amitoses probably take place in all possible planes, the error due to the above factors is no doubt a considerable one.

A fourth difficulty needs to be considered, namely, the possibility that the larvæ studied were abnormal. To eliminate errors due to this source I used more than one embryo in each of the stages represented in Fig. 9, except the first two, of which no greater number was available. Since the argument, as the sequel will show, does not hinge on individuals, but on a comparison of the first half of the developmental period considered, with the second half, the scarcity of early stages is compensated for. Thus

the results are actually based on three larvæ of the cannibal period, and on four of the post-cannibal period though many others were used for comparison.

A final difficulty not at all peculiar to *Fasciolaria*, but to be expected wherever amitosis occurs, is this : How can one tell that what seems to be an amitotic division is really such? Since in amitosis there occur none of the striking changes that characterize mitosis, it is, as Hertwig ('98) has pointed out, impossible to be sure that direct divisions are going on unless one can find all possible stages in the process. The mere lobulation of nuclei is not sufficient. I believe that Fig. 7 is an answer to the criticism which neglect of Hertwig's warning might justify. Of course, many of the nuclei there pictured would not have been included in the same plate with those which I cannot doubt are amitotic, had I not found the latter. Given stages however which it is impossible to interpret in any other way, it seems mere pedantry to exclude all of the others which taken by themselves, would either not be convincing, or to the casual observer, might not even suggest amitosis. Had it been impossible for instance to find all of the intermediate stages between a resting nucleus and a late metaphase, I doubt very much whether anyone totally ignorant of the process of mitosis would be able to assert that the latter stage had been derived from the former. The initial and final conditions however are safely interpreted in terms of the intermediate stages that have been found, and every step in the process is illuminated by every other step. However, I have chosen to err on the safe side, and while Fig. 7 includes all of the different nuclear forms met with, in the actual counts only nuclei like 6, 7, 8, 9, 10, 11, 12 and 13, were included. None of the nests, such as 16 and 17, were counted, nor the elongated forms, like 18 and 19, from which the nests may have been derived. Even nuclei as close together as 14 were not included, nor such as 15 in which the cell boundary enclosing them could, as is sometimes the case, be distinctly traced.

Summing up the effects which all of these difficulties and their evasion have on the final result, I think it may be justly said that the incompleteness of many of the sections is without significance; that the complete elimination of the temporary cells

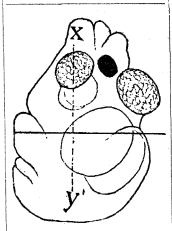
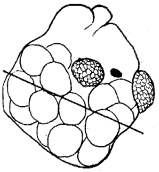
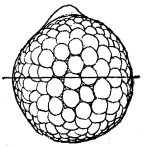
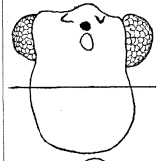
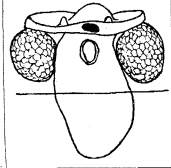
Stage.	Condition.	Sections.	Entoderm.	Nuclei.	Mitoses.	Amitoses.	Cells.
I.		38	Cuboidal *a*	339	17	21	20 *b*
II.		12	"	143	0	0	20
III.		65	Elongated	916	2	42	20 *c*
IV.		15	Cuboidal	589	1	28	57
V.		51	"	1,162	0	41	93
Totals				3,149	20	132	

FIG. 9. The first column at the left contains the numbers applied for purposes of description to the several stages used; the second column contains outline drawings illustrating the condition of the stages employed; the third the number of sections studied; the fourth a statement of the condition of the entoderm; the fifth the number of nuclei counted; the sixth the number of mitoses seen; the seventh the number of amitotic divisions registered; and the eighth the number of entodermal cells present in a section cut in the plane of the heavy line which is drawn through each picture of the larval stages in the second column. Wherever number or other statements are based on inference or deduction, this fact is indicated by a small letter which refers to a foot-note, which in turn refers, if necessary, to the page where the evidence on which the inference or deduction is based, is given in full. (*a*) See Fig. 2 and page 238. (*b*) Estimated; see Fig. 2 and page 238. (*c*) Estimated; see page 239.

involves also the elimination of a considerable number of permanent ones; and that the fact that mitoses can be cut in more planes, and also into a greater number of sections in any plane, than amitoses, and still reveal their true nature, increases greatly the percentage of indirect divisions in the determinations of the relative frequencies of these two forms of division. The fact that amitoses are much harder to recognize than mitoses, and that I counted as direct divisions only those that seemed to me unquestionable cases, also helps to increase the relative frequency of mitosis in the final determinations. It follows therefore that the methods employed give a maximum of mitoses and a minimum of the process that I interpret as amitosis.

The Relative Frequency of Mitosis and Amitosis.

The main results of my work are graphically illustrated by Fig. 9. There are arranged in tabular form, outline drawings of larvæ in the stages of development used, and on a line with each one are the number of sections on which the determinations are based; a statement concerning the condition of the entoderm; the number of nuclei actually counted; the number of mitotic divisions found; the number of amitoses registered; and the number of cells either actually found in the transverse planes indicated on the drawings, or inferred to be present there from evidence which will be given in detail for each case in which inference replaces actual counting.

Analysis of this table reveals several interesting facts. In stage I. for example, there is a higher percentage of mitoses, and of course a lower percentage of resting nuclei than in any of the other stages. Three years ago when I had worked out the relations between direct and indirect nuclear division in the entoderm, I had come to the conclusion that in the early stages the divisions in this tissue were predominantly mitotic whereas in the later stages the reverse was true. My notes in some way were lost but on repetition the same result, as the table shows, appeared. I thought at that time that the numerous amitoses in the later stages were connected entirely with digestion, and were of no significance in the formation of the definitive entoderm. Although not published in any journal I expressed this view in a

paper read before the Research Seminar at Wood's Hole in the summer of 1905. This view may still appeal to some as valid, but I think that certain facts then unknown to me point strongly in the opposite direction. While it is true that during the early stages of cannibalism the increase in the size of the embryos is mere stretching due to the ingestion of eggs, such stretching is not all that happens in the larvæ. It will be recalled that the entoderm in stage I. is a pretty thick layer in which cell boundaries are obscure. An examination of the layer however will show that if cell boundaries were distinct, the cells would be cuboidal, or rectangular or at most diamond-shaped (see Fig. 2). After the larvæ have taken on the form of stage III. the entoderm, as Fig. 3 shows, has an entirely different appearance, and the distinct outlines of the cells show that these instead of having the shape of cubes or rectangles, or diamonds, are spindle-shaped, and provided with long processes that by fusion with corresponding elongations from neighboring cells form a continuous membrane. I think that no special argument is necessary to support the view that the transformation undergone by the entoderm cells when the larvæ pass from stage I. to stage III., is the direct result of stretching. Such an effect is to be expected. This condition however is not final. The elongated entoderm cells soon lose the characteristics which they exhibit in stage III. and return to a condition more nearly like that in stage I., except for two general differences: boundaries are more distinct than in the younger entoderm, and regional differentiation is observable.

These metamorphoses are of great importance. If the larvæ decreased markedly in size during the later stages of their development, and as the result of such shrinkage approached the size of the pre-cannibals, the conversion of the spindle-shaped cells of stage III. into the cuboidal cells of stages IV. and V. could be attributed to this cause: to decreased stretching. The idea that the later stages in development might be smaller than the earlier, is somewhat bizarre when viewed in the light of our knowledge of ontogeny in general, for the exact reverse is law. Nevertheless such decrease in *Fasciolaria* embryos is quite easily conceivable as the size depends on what the larvæ contain. If metab-

olism were very intense, and the swallowed yolk were very quickly used up, instead of remaining in the digestive tract for five or six weeks as it actually does, the elimination of waste products might be rapid and great enough to bring about an actual decrease in the size of the later stages. Such shrinkage would indeed occur were the effects of the digestion of yolk and of the elimination of wastes not more than compensated for by four factors, three of which obtain in every individual, whereas the fourth is operative only in the majority of cases. In the first place, after the period of cannibalism, the larvæ actually increase in size by ordinary growth; in the second place, even after completely filled with eggs, they continue to inflate themselves with the albuminous material in which they float; and thirdly several days after ingestion many of the eggs lose their pellicles, and since the yolk granules are large, very firm, and vary considerably both in size and shape it is to be expected that they would take up more room than when neatly packed, as they are, in the intact ova. In addition to these factors of enlargement, one very remarkable one operates in so many cases, that it may be called a matter of common occurrence. In every capsule, practically, some of the cannibals in stage III. burst, and in those egg-cases in which only two or three larvæ in later stages are found, the majority of cannibals have broken. In these instances the surviving larvæ are invariably larger than those in the more populous capsules. Experiments have shown that a fully gorged cannibal, which under other conditions would have ingested no more eggs, will double the number it contains if the food supply is increased. From these experiments, as well as from the observation that where embryos are below the average in number they are above the average in size, and the further observation that bursting accidents occur in practically every capsule, it follows that after the stretching which transforms stage I. into stage III. has occurred, a further increase in size depending upon the factors mentioned takes place.

During the particular period of development now under consideration the activities of the entoderm are such that in spite of the stretching due to all of the causes mentioned, the cells of the inner layer change from the spindle-shape to the cuboidal. The

only way in which such a change can conceivably take place seems to me to be by a very rapid increase in the number or the size of the cells. An increase of the former sort can very readily be noticed during the period when the spindle-shaped cells of the early pre-cannibals (Fig. 1) change to the "cuboidal" cells of the young cannibals (Fig. 2). If a similar increase takes place when the fully gorged cannibals transform into veligers one should be able to find histological evidence of it. While the original transformation may be accounted for on the basis of 17 mitoses per 339 nuclei, the second transformation, if mitosis is the only method of division in normal cell differentiation, must be accounted for on the basis of 1 mitosis in 1,751 nuclei. This single case is absolutely the only indication of mitosis, that careful and frequently repeated search through stages IV. and V. has revealed. On the other hand I found in the same sections 111 cases of what I interpret as undoubted instances of amitosis. If the amitoses do not account for the increase of cells needed to explain the change from the spindle to the cuboidal shape, I doubt very much if the all but total absence of mitosis accounts for the facts. It is necessary to conclude therefore, either that the only form of division seen in any quantity is responsible for the assumed increase in cells, or that these enlarge and become comparatively crowded.

The crucial question then is, which of these two explanations is correct? Do the cells become crowded because they increase in size, or in number or for both reasons? The drawings show plainly that the dorsal cells do increase in size during the metamorphosis; they also show that this is not true of the ventral cells. In addition to the enlargement of the liver cells it can be shown that actual increase in the number of cells present, is a factor bringing about the change from spindle-shaped to cuboidal cells.

Absolutely faultless series, or strictly comparable sections are in several cases unavailable. Of stage I. for example, I have no transverse sections, but a study of the differences between the long axis and the short one of this larva makes 20 cells in a transverse section midway between the extremities of the longer axis, a safe estimate (see Fig. 2 and stage I., Fig. 9). In stage

II. actual counting of complete sections in an approximately comparable region, viz.: midway between the extremities of the long axis, gave 20 as the number of cells, whereas for stage III., 15 was the average of five incomplete sections taken near the plane of the equator. It is practically impossible to secure entire sections through larvæ in this condition on account of the thinness of both the body-wall and the wall of the digestive tract, neither of which is thicker, in many regions at least, than the pellicles around the ingested food-ova. In the sections from which the particular estimate now under consideration was made, one third of the circumference was incomplete; as there were fifteen cells in the other two thirds, I assumed that the torn region represented a distance which in the entire embryo was covered by five cells, an assumption justified by the study of other sections.

A comparison of stages II. and III. may suggest at first sight that the younger embryo should have fewer cells than the older one, since the latter contains so much more material than the former. Professor Osborn says that there are not enough cells in stage III. to enclose the food-ova. This however is a mistake; there are enough cells, only in order to "cover the ground" these are stretched almost beyond belief. Indeed the elongations are frequently as attenuated as the delicate projections so characteristic of mesenchyme cells.

The number of entoderm cells in stages IV. and V. was determined in complete sections, for the body-wall as well as the wall of the digestive tract have thickened so much in these older embryos that it is a comparatively easy matter to section them without injury. As the table shows, 57 cells is what I found in the younger of the two oldest stages and 93 cells in the oldest of all of those considered.

Granting for the sake of argument that the number of entoderm cells in the earlier stages is twice as great as my determinations indicate, the later stages would still show double the number of cells in corresponding regions. Fig. 2 shows that such an error is impossible. I am certain that the figures actually given are much nearer the truth and that instead of having twice the number of entoderm cells, the later stages of the de-

velopmental period considered have four times as many. As the period during which this increase chiefly occurs exhibited but one mitosis among 1,751 nuclei, the conclusion is practically forced on one that amitosis is the method of cell multiplication that obtains in the entoderm.

This conclusion however must be critically tested. Is there any possibility that after all one mitosis among 1,751 nuclei is enough to account for the facts of growth? This question can, I think, be definitely answered in the negative.

The time taken by a larva in stage III. to change into stage IV. is 13 days ('05). During this period, according to the determinations, the number of cells in the transverse planes under consideration increases from 20 to 93, an addition of 73 cells. Let us assume for the sake of argument that a complete mitosis, beginning with a resting mother nucleus and ending with two resting daughter nuclei can be accomplished in one minute. As $\frac{1}{2\,0}$ per cent. of the nuclei are dividing mitotically, it follows that in one minute .0005 mitosis occur. In 2,000 minutes therefore one complete mitosis would take place. Since 2,000 minutes equal 33 hours, it follows that once in this number of hours an entoderm cell would divide. Now the developmental period under examination endures 13 days, or 312 hours. If therefore one division takes place every 33 hours it follows that 9 such cleavages would occur in the 13 days. As the larva has "20" cells to begin with, the first division would raise this number to 21; the second to 22; and the ninth to 29. Thus if mitosis occurs at the determined rate of $\frac{1}{2\,0}$ per cent., and at the assumed speed, 9 new cells would have been produced. The actual counts show that 73 cells are added. Even if we double the speed and assume that a mitosis can be completed in 30 seconds there would still be a disparity of 55 cells. If this reasoning is correct, mitosis occurring with the frequency actually determined, is totally insufficient to account for the observed facts of growth.

One chance however remains. It is possible that my determinations of the frequency of mitosis during this developmental period are misleading; that I missed the epidemics of division, three of which would more than explain the facts, for if all of the 20 cells in stage III. were to divide at once the number would

be doubled ; the second epidemic would yield 80 cells and the third 160. A less severe epidemic, one having more probability in fact, might exactly account for the approximately fourfold increase observed.

The assumption that epidemics of mitosis occur, but have been overlooked, is unfortunately without foundation. In the larvæ used for the determinations of the relative frequencies of mitosis and amitosis, as well as in the many others used as checks in working out the more strictly embryological details, I have never observed any indications of such epidemics. Similar indications seem also to have escaped Osborn.

It is impossible on the basis of such negative evidence as is available to assert dogmatically that they do not occur. My results however have some significance in this connection. A comparison of stages III., IV., and V., shows that I found 2 mitoses, 42 amitoses, and 20 cells in stage III.; 1 mitosis, 28 amitoses, and 57 cells in stage IV.; and 0 mitosis, 41 amitoses, and 93 cells in stage V. It might be asserted that the divisions which account for the increase from 20 to 57, took place during an epidemic of mitosis at some time between stages III. and IV. It might be claimed also, that the increase from 57 to 93, had come about as the result of a similar epidemic between stages IV. and V. These stages were selected because their external characteristics mark definite steps in the acquisition of the adult body form. Stage IV. is approximately a half-way station between stages III. and V. It is important therefore that in other respects also the larvæ should be half-way between the two extremes, for this is at least an indication of an even tenor in the rate of all of the developmental processes. Were growth spasmodic and not uniform, it would be very curious that the number of entoderm cells in a corresponding cross-section of the "half-way" larva should be 57, for the mean between 93 and 20 is 56.5.

The view that growth is uniform in rate gains in validity when we consider the percentage of indirect and direct divisions which occur during this crucial period. For stage III. the former is .2 per cent., the latter 4 per cent.; for stage IV., the former is .1 per cent., the latter, 4 per cent.; whereas, for stage V., we have 0

per cent. of mitosis, and 3 per cent. of amitoses. Allowing for errors, there is practically no fluctuation in the frequency of either mitotic or amitotic division in these three stages of development. Since this is true, to say that the rate might have been very different between stages III. and IV., and again between stages IV. and V., may be true, but is supportable by neither facts, nor probability. Indeed, it is not going too far to say that the percentages as well as the number of cells found, indicate the exact opposite, namely: that in this tissue, at this particular period of development, mitosis and amitosis occur at constant frequencies.

Considering the table as a whole, it follows that of 3,340 nuclei, a little less than .6 per cent. exhibited mitotic figures. If my interpretation of what constitutes amitosis is correct, then a little over 87 per cent. of all divisions are direct, whereas only a trifle more than 12 per cent. are mitotic. As I have pointed out before, these figures undoubtedly contain a large error due to the fact that early as well as late stages in amitosis are not sufficiently well marked to enable one to decide whether they belong into this category or into that of the resting nuclei. As all doubtful cases were relegated to the latter group, I feel confident that 87 per cent. represents the minimum of amitosis, and that in all probability the direct divisions are more frequent. In view of this I think that the conclusion is justified that amitosis is the chief mode in which the nuclei and cells increase in number. Of the two alternatives which these results allow, one, the possibility of epidemics of mitosis, is not only unfounded, but improbable; the other, namely, that a four-fold increase in cells can be accounted for on the basis of 1 mitosis in 1,751 nuclei, involves an absurdity.

Discussion.

I do not propose to enter at this time into an elaborate discussion of either the literature on amitosis, or of the theoretical questions on which direct nuclear divisions are thought to bear. The former has been very ably done by other writers, notably Henneguy ('96) and Wilson ('02), the latter I shall do after I have accumulated more data. The belief that in the entoderm of *Fasciolaria* we have an instance in which amitosis plays an important if not the chief part in the differentiation of a definitive

tissue, can however be supported by several references in the literature, and these I shall at least mention.

I have already referred to the work of Meves ('91) on the spermatogenesis of the salamander. In this well-known paper evidence is brought forward which shows that in the spermatogonia amitotic divisions take place during the fall, and that these succeeded in the following spring by the usual maturation phenomena, are part of the cycle of a normal organism. Wheeler ('89), in his paper on the embryology of *Blatta germanica* and *Doryphora decemlineata* has reached similar conclusions. Thus in *Blatta*, cells originate in the center of the ovum by mitosis. These cells are amœboid, and wander to the surface of the egg where they flatten out. "The cells which have reached the surface and are much scattered over the roof-shaped ventral face and the adjacent portions of the lateral faces commence dividing longitudinally, not by karyokinesis, as heretofore, but by akinesis." "My observations," continues Wheeler, "tend to show that all of the future divisions in the formation of the blastoderm, and those subsequently undergone by the serosa, are akinetic, the densely coiled chromatin filament remaining inert and the divisions taking place by a constriction which often produces two daughter nuclei of very unequal size. I emphasize the fact that these forms of division could not have been produced by the reagents, as the eggs were hardened in picro-sulphuric acid or simple alcohol, which in younger and older eggs preserve the karyokinetic figures of the cleavage nucleus and its immediate descendants with great clearness." From this it follows that the cells that make up the germ-layers from which the definitive cells of the body come, are all descended from cells which at an earlier period of development divided by amitosis.

Frenzel ('92) came to the conclusion that amitosis plays an important rôle in the regeneration of the intestinal elements in the crustaceans, and insects, for he claimed at first never to have found any indirect divisions. As Henneguy ('96) pointed out after Frenzel himself had corrected the mistake the conclusion that mitosis does not occur in the cells in question is undoubtedly incorrect, but the fact that the digestive tract in certain arthropods can be studied carefully without revealing any mitotic divisions,

shows at least that these must be rare. The same thing may be said of embryonic tissues in general, as Child has emphasized. Who has not been struck by the comparative scarcity of mitosis in tissues which are known to grow with great speed?

As implied in the introduction to this paper, whether amitosis plays a part in normal cell-differentiation, and whether direct divisions may intervene between indirect ones, without inhibiting further differentiation are really two distinct questions. In practice however it is impossible to keep them separate, for if amitosis does play a rôle, it does this in a normal tissue, and it is characteristic of normal tissues that their component cells at some time exhibit mitosis. The results both of Meves and of Wheeler offer cases in point. The same is true of Child's work. In *Moniezia* also cells which are part of an apparently normal cycle divide at one time amitotically (oögonial and spermatogonial divisions) and later mitotically (maturation divisions). Similarly after fertilization, the first cleavage of the egg is accompanied by a typical mitosis, whereas the later cleavages may be amitotic. Since the cells of the cleavage period are the ones from which the definitive structures of the adult come, it follows that amitosis plays a part in normal cell differentiation.

Neither *Moniezia*, Child's form, nor *Fasciolaria* are ideal animals to work upon, for aside from the mere matters of technique which in one of them offer considerable difficulty, both of these forms exhibit in the tissues studied (entoderm; ovary; testis) degenerating cells, and a certain number of mitotic divisions along with the amitotic ones. The possibility therefore exists that the indirect divisions are the really important ones, whereas the amitoses are physiological, and of no consequence in a genetic sense. According to Wheeler *Blatta* must be ideal for "all of the future divisions of the blastoderm and those subsequently undergone by the serosa are akinetic." Apparently here there is no chance of a mistake. In the absence of other forms equally well adapted for our purposes, there is only one thing to do — to measure as accurately as possible the frequency of the direct and indirect divisions in a tissue, and then on the basis of these measurements to see if the facts of growth that need explanation can be explained when one or the other of the two forms of division is ruled out.

This is what I have tried to do in the case of *Fasciolaria*, and what seems to me ought to be done in other forms. Merely stating that mitosis and amitosis occur, without also stating how frequently, does not meet the requirements of the problem.

If his interpretation of the life history of *Amœba proteus* is correct Calkins ('07) has advanced an absolutely conclusive case in which direct nuclear division is a link in a normal life-cycle. Calkins believes he has found evidence adequate to show that in *Amœba proteus* an asexual period is succeeded by a sexual one inaugurated by amitotic multiplication of the nucleus. The "primary nuclei" thus formed fragment and change to minute granular "secondary nuclei." The secondary nuclei later conjugate giving rise to the "fertilization nuclei"; "in these the fused karyosomes fragment to form finely divided chromatin (it is strictly speaking, not a chromidium for it is entirely intranuclear), while a vacuole forms in the interior; this vacuolated fertilization nucleus becomes a center of multiplication (equivalent in every way to a sporozoön sporoblast); by accumulation of these fine chromatin granules the peripheral or 'tertiary' nuclei are formed; the tertiary nuclei, surrounded by a minute bit of plasm, grow into the pseudopodiospores observed by Scheel (hypothetical); these young pseudopodiospores break away from the parent cyst and develop into young amœbæ fromerly known as *Amœba radiosa*, and these in turn develop into the ordinary *Amœba proteus* of pond and laboratory." If this represents truthfully the life cycle of *Amœba*, we have at least one conclusive case in which amitosis cannot be ruled out, for here there are no mitoses. Neither are there any degenerating cells to cast their shadow of suspicion on the other cells. Equally conclusive cases can hardly be hoped for among the higher animals, although what seems to be true for *Amœba*, may be also true of multicellular forms. If it proves impossible to establish these facts with mind-compelling certainty, further investigation should be able at least to endow them with a degree of probability amounting to a practical demonstration.

Summary.

1. During the period of cannibalism, the entoderm of *Fasciolaria* becomes first spindle-shaped, but later as regional differentiation occurs, the cells become cuboidal.

2. The first change can be accounted for by the stretching which the larvæ undergo; the second change is explained by a fourfold increase in the number of cells found in transverse sections through the middle of the digestive tract.

3. During this period of cell increase there was found a maximum of one mitotic division in 1,751 nuclei.

4. During the same period of development, there was found a minimum of 69 amitotic divisions.

5. From this it follows that during the period of most active cell multiplication more than 1 per cent. of all divisions is mitotic and more than 98 per cent. are amitotic.

6. Since there were found during the pre-cannibal, the cannibal, and the post-cannibal periods, 152 cases of what is interpreted as nuclear division, and since of these 20 were mitotic, it follows that during the entire developmental period considered a little over 13 per cent. of all the divisions were mitotic and a little less than 87 per cent. amitotic.

7. Therefore the conclusion is reached that amitosis plays in this instance an important, if not the chief part in the differentiation of a definitive tissue.

8. Of the two alternatives which might be suggested, one, that unobserved epidemics of mitosis account for the facts, is not only without foundation, but is improbable; the other, that a fourfold increase in cells can be accounted for on the basis of 1 mitotic division per 1,751 nuclei involves an absurdity.

Postscript.

By an oversight I have omitted a reference to Professor Hargitt's observations on the occurrence of amitotic divisions in the development of certain cœlenterates. In his paper entitled "The Organization and Early Development of the Egg of *Clava leptostyla* Ag.," Biol. Bull., Vol. X., Hargitt says: "During the early cleavage, even up to the sixteen-cell stage, no evidence of mitosis has been found." Similar experiences were met with in studying the development of *Eudendrium* and *Pennaria*, and Professor Hargitt adds: "as facts multiply . . . cytologists will be forced to take cognizance of this form of cytogeny and give it something more than a merely incidental place in cellular activities."

Quite recently, in fact after the present paper had gone to press, I received a reprint of the memoir "On *Turritopsis* (McCrady)," *Proc. Bost. Soc. Nat. Hist.*, Vol. 33, No. 8, by Professors Brooks and Rittenhouse. These authors record the occurrence of direct nuclear divisions during the development of *Turritopsis*, and incline toward the conception of Flemming and Ziegler, that amitosis is connected with cellular specialization or degeneration, as the process is most abundant in *Turritopsis* shortly before cell boundaries disappear and the embryo becomes transformed into a syncitium. As the adult is derived from this syncitial embryo it is not unreasonable to consider the amitoses in question as developmentally significant parts of a normal cycle, as stages in the establishment of adult definitive tissues, a view supported by the evidence recorded in the preceding pages.

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